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Changes in field dose-response curves for DMI and QoI fungicides against *Zymoseptoria tritici*, related to laboratory sensitivity phenotyping and genotyping assays

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Abstract

BACKGROUND: Insensitivity of *Z. tritici* to de-methylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides has been widely reported from laboratory studies, but the relationships between laboratory sensitivity phenotype or target site genotype and field efficacy remain uncertain. This article reports field experiments quantifying dose

response curves, and investigates the relationships between field performance and *in vitro* EC_{50} values for DMIs, and frequency of the G143A substitution conferring QoI resistance.

RESULTS: Data were analysed from 83 field experiments over 21 years. Response curves were fitted, expressed as percentage control, rising towards an asymptote with increasing dose. Decline in DMI efficacy over years was associated with a decrease in the asymptote, and reduced curvature. Field ED_{50} values were positively related to *in vitro* EC_{50} values for isolates of *Z. tritici* collected over a 14 year period. Loss of QoI efficacy was expressed through a change in asymptote. Increasing frequency of G143A was associated with changes in field dose response asymptotes.

CONCLUSION: New resistant strains are often detected by resistance monitoring and laboratory phenotyped/genotyped before changes in field performance are detected. The relationships demonstrated here between laboratory tests and field performance could aid translation between laboratory and field for other fungicide groups.

Key words: septoria tritici blotch, *Zymoseptoria tritici* (Desm.), azole, QoI, fungicide sensitivity

1 INTRODUCTION

Fungicides that block metabolic pathways by acting on a single target

enzyme are prone to loss of efficacy due to resistance evolution in pathogen populations. Since the introduction of fungicides to cereal production, four single-site acting fungicide groups have provided control of *Zymoseptoria tritici* (causal organism of septoria tritici blotch; STB), namely: benzimidazoles, sterol demethylation inhibitors (DMIs), quinone-outside inhibitors (QoIs) and succinate dehydrogenase inhibitors (SDHIs). Resistance to benzimidazoles due to a mutation resulting in the E198A beta-tubulin target site change developed rapidly in *Z. tritici* populations in the mid-1980s and resulted in poor control.[1] QoI fungicides demonstrated a high level of activity against STB when introduced and were widely used in the UK by 1998.[2] Resistant strains carrying a mutation underlying the G143A amino acid substitution in the cytochrome *b* target site [3] were first detected in the UK in 2002, although retrospective studies found G143A in 2001 leaf samples from QoI treated field plots [4]. In successive years, 2003, 2004 and 2005, the frequency of resistant isolates in the UK increased rapidly [5] resulting in a severe loss of field efficacy where QoIs were used alone. The frequency of QoI sensitive strains was below 5% in UK and Irish populations sampled in 2016 (Fraaije; Kildea, unpublished data). Isolates carrying cytochrome *b* F129L alleles have been detected in Ireland [6] but these strains have not become widespread due to lower levels of QoI resistance in comparison with G143A strains. Resistance against benzimidazoles and QoI can be described as being of a 'qualitative', 'disruptive shift' or 'single step' type, whereby strains carrying a specific target site mutation of large phenotypic effect cause the population to become bimodal and rapidly dominate the pathogen population.

The DMI fungicides, in particular the azoles, have been used widely to

control *Z. tritici* since their introduction in the 1970s.[7] *In vitro* sensitivity testing of field *Z. tritici* isolates with the DMIs epoxiconazole and prothioconazole over the last 12 years has shown progressive increases in their mean EC_{50} values.[8] Decreased sensitivity has been associated with alteration of the CYP51 target protein, overexpression of the sterol 14α-demethylase gene *CYP51*, and enhanced efflux.[8,9,10,11,12,13,14,15,16] DMI resistance can be described as being of a 'quantitative', 'progressive' or 'slow shifting' type. The translation between laboratory tests and field performance is likely to differ between qualitative and quantitative types of resistance.

For quantitative resistance, such as to the DMIs, changes in *in vitro* EC_{50} values should be associated with changes in field efficacy; particularly when laboratory EC_{50} values increase substantially, indicating that the field population has a high frequency of moderately or highly resistant strains. However, demonstrating a relationship between laboratory sensitivity phenotype and field performance has proved difficult. Reductions in *in vitro* sensitivity were reported in France and across Europe with epoxiconazole,[17] but these did not correlate with any change in efficacy in field trials. In 2013 the Fungicide Resistance Action Committee (FRAC) reported that DMI field performance was good when used according to the manufacturers and FRAC recommendations.[18] Despite anecdotal evidence of declines in field performance, no study has quantified a reduction in field efficacy for either epoxiconazole or prothioconazole that consistently reflects the *in vitro* data.

There are two main explanations for the lack of a clear relationship between laboratory and field performance: Firstly, *in vitro* sensitivity phenotyping can detect small shifts in EC_{50} values, which may be too small to

change efficacy at doses typically used in the field, even if such strains are at high frequency. Secondly, field experiments are subject to many extraneous variables that add uncertainty to measurements of efficacy, even if they are conducted to a consistent protocol across sites and seasons. Error variation is caused by many environmental, treatment and assessment factors, such as differences in when fungicide treatments are applied (which can be weather dependent) relative to when specific culm leaf layers emerge and become infected. The efficacy measured is highly dependent on the relationship between application timing, leaf emergence, infection events, the leaf layers that are subsequently assessed and the timing of those assessments.[19]

The field experiments described here tested fungicide performance in the UK and Ireland, by a standard protocol, over two decades. The analysis addressed some of the sources of extraneous variability described above, in order to improve the likelihood of detecting underlying relationships between laboratory and field performance. Field performance was related to data from laboratory assays of *Z. tritici* isolates collected at a single location.

Changes in field performance of the DMI and QoI modes of action, were quantified by fitted dose response curves of the form $y=a+be^{kx}$, where y = % disease, x = dose, k quantifies the curvature, a the asymptote and $a+b$ the untreated severity.[19,20] From first principles, the relationships between changes in laboratory sensitivity and changes in field dose response curves might be expected to differ between qualitative and quantitative resistance.

For example, with quantitative resistance against DMIs, estimated field ED_{50} (defined here as the dose which reduced severity to half of the untreated severity) values can be compared with laboratory mean EC_{50} values. As

mutant variants arise resulting in a progressively wider and more shifted population distribution of sensitivities, the field dose response would be expected to become less strongly curved, resulting in changes to the curvature parameter *k*. This is because, if many variants are slightly or moderately insensitive, control at lower doses would be more strongly affected than control at higher doses. However, if a proportion of variants with high levels of insensitivity also arise, control at doses at, or close to, the maximum permitted dose may also be affected, resulting in a change of asymptote *a*, as a proportion of the pathogen population cannot then be controlled.

For the contrasting case of qualitative resistance against QoIs, isolates are either sensitive or highly insensitive, so the hypothesis tested was that changes over years in the resistant proportion of the pathogen population would be expressed in the field as a change in the asymptote of dose response curves (representing the proportion of disease which cannot be controlled). Understanding the relationships between these laboratory and field variables could help with the future translation of phenotypic or genotypic changes into field efficacy for new modes of action.

2 EXPERIMENTAL METHODS

2.1 Trials

Eighty three field experiments were conducted between 1994 and 2015. In each year (with exceptions in 1999 and 2000) between two and seven trials tested fungicide efficacy on *Z. tritici*. DMI (epoxiconazole and prothioconazole) and QoI (azoxystrobin, trifloxystrobin and pyraclostrobin)

fungicides were applied once at one of four doses: 25%, 50% 75% and 100% of the maximum permitted dose (between 1994 and 1998); and 25%, 50%, 100% and 200% of maximum dose (between 2001 and 2015) (Table 1). One or two untreated control plots were included in each replicate. Trials were sited at locations in England, Scotland and the Republic of Ireland known to suffer damaging epidemics of *Z. tritici* in most seasons. Cultivars susceptible to STB but resistant to other diseases were selected to reduce confounding effects from other diseases. Fungicides were applied when final leaf 1 (the flag leaf on the mature culm) was fully emerged, or leaf 2 (the leaf below) fully emerged or leaf 3 fully emerged, depending on the experiment. Fungicides were applied by hand-held sprayer in 200-300 litres water ha^{-1} at 200-300 kPa pressure with a medium spray quality. Minimum plot size was 20 m^2 with three replicates, using either complete randomized blocks, or incomplete blocks using an alpha design.[20]

2.1.1 Disease assessments

Foliar diseases were assessed approximately 21 and 42 days after spray application. Ten shoots were taken at random from each plot and each leaf layer scored for % leaf area affected. Leaf layers were included in the analysis where mean untreated *Z. tritici* severity was between 3% and 70%, and a significant response to fungicides was observed. The upper and lower limits on severity were imposed, based on judgement of experienced disease assessors, as very low and high severities are difficult to assess accurately.

Severity data from a leaf layer in an experiment was classified as representing 'protectant' efficacy if the leaf had just emerged or was emerging

at the time of treatment. Severity data from a leaf layer in an experiment was classed as representing 'curative' efficacy if the leaf layer had fully emerged 10 days or more prior to treatment. Curative data was included in the analysis from up to two leaf layers below the leaf which was fully expanded at the time of treatment, as infections on older leaf layers further down the canopy were likely to have already expressed disease symptoms, or be close to expressing symptoms, at the time of treatment.

As the date of leaf emergence relative to the date of fungicide application can only provide an approximate guide to whether the leaf was likely to have latent infections at the time of treatment (and hence, whether the treatment was applied in a predominantly 'protectant' or 'curative' situation), an additional method of categorising the severity data was used, based on assessments from replicated 'control' plots in each experiment that were treated with 500 g/ha of chlorothalonil (as commercial product 'Bravo 500'; Syngenta) which is a protectant fungicide. If the control by chlorothalonil exceeded 60% efficacy on a leaf layer previously categorised at 'curative', then it was assumed that (due to environmental conditions) little infection of that leaf layer had occurred between emergence and treatments. Hence, the severity data from that leaf layer in that experiment was re-categorised as 'protectant' for the analysis. In the same way when control from chlorothalonil was found to be between 40% and 60%, on an 'eradicant' leaf, severity data was classified as mixed rather than eradicant, and was excluded from this analysis.

2.2 *In vitro* sensitivity testing and genotyping

2.2.1 DMI sensitivity testing

Ideally, to compare *in vitro* and field sensitivity, EC_{50} values should be obtained from samples of *Z. tritici* from multiple locations (including the locations of the field experiments) in a long run of consecutive years. Due to resource limitations, such datasets do not exist in the public domain. Instead, *in vitro* sensitivity data were used from sampling at one location in the UK in a sub-set of the years between 2003 and 2015. To quantify variation in sensitivity across sites (for comparison against variation across years), data were obtained from samples from a range of UK locations taken in 2013 and 2015. Strains of *Z. tritici* were isolated and tested for fungicide sensitivity using optical density measurements of fungal spore growth in 96-well microtitre plates according to the method described by Cools *et al*. (2012). Before dilution in media, fungicides were dissolved in dimethylsulphoxide (DMSO). All wells, including untreated, were adjusted with DMSO (final concentration 0.2% v/v in each well). *In vitro* EC_{50} values[8] of *Z. tritici* were measured for epoxiconazole and prothioconazole from isolates collected from the same location in the UK (Rothamsted, Harpenden, UK) between 2003 and 2015. Prothioconazole-desthio (an active metabolite) was used as a surrogate for prothioconazole. In 2013, samples of populations were obtained for *in vitro* sensitivity analysis from Cambridgeshire, Shropshire, Hampshire, Herefordshire (2 populations sampled), Scotland, Kent and Hertfordshire (Rothamsted). In 2015, samples of populations were obtained from Cambridgeshire (3 populations), Yorkshire (2), Herefordshire (2), Gloucestershire, Kent, Norfolk, Lincolnshire, Scotland, Hampshire and Hertfordshire (Rothamsted).

2.2.2 QoI G143A genotyping

Isolates carrying the G143A QoI target site mutation have very high resistance factors, resulting in bi-modal population distributions of EC_{50} (comprising sensitive and insensitive sub-populations). A mean EC_{50} for a population is not, therefore, a useful metric for the sensitivity of a population. The frequency of G143A mutants in a population provides a metric which is more likely to relate to field performance, as the frequency represents the proportion of the pathogen population which will not be effectively controlled at any field dose. As reported by Fraaije *et al*., 2006 [21] *Z. tritici* isolates were obtained, and genotyped for G143A, from untreated fields of winter wheat at Rothamsted, UK, at the start of each seasons in consecutive years from 2001 to 2006, and in 2015.

2.3 Data management and statistics

Percent *Z. tritici* severity values were logit transformed and means calculated for each experiment. Means across experiments were calculated after logit transformation by linear mixed model using REML (residual or restricted maximum likelihood), with fixed (treatment) and random (trial and year) effects. Fungicide dose response curves were calculated from back transformed data using the exponential function $y=a+be^{kx}$, where y = % disease, and x = proportion of full label rate.[19,20] Results were analysed for QoI and DMI active substances for which several years of data were available (see Table 1), using GenStat Release 13.3.[22]

Dose response curves were fitted to percent *Z. tritici* for the range of fungicide doses and converted into percent control to allow comparisons of efficacy between sites and seasons despite differences in untreated severity.

Possible effects of untreated severity on percent control were determined.

3 RESULTS

Dose response data were obtained every season for epoxiconazole (from 1995) and prothioconazole (from 2001) until 2015 (Table 2), with the exception of 1999 and 2000 (when no experiments were conducted). With few exceptions, each experiment produced dose response data which reflected predominantly curative control and protectant control, from assessments of lower and upper leaves of the crop canopy respectively. Exceptions to this were in 1998 when only curative dose response data were available; and in 2010 and 2015 only protectant dose responses were obtained (Table 2). Chlorothalonil, as a control, was tested at 0.5 label rate in 1995 to 1996 and 2003-2015, and there was no indication of a change in efficacy over this period. The performance of the three QoI fungicides was quantified from between one and six dose responses in each of the five years reported here.

3.1 QoI efficacy

In 2001 and 2002, the QoI fungicides gave highly effective control of STB (Figure 1). The dose response curves for trifloxystrobin and pyraclostrobin were highly curved, with percent control achieved at a quarter or half the maximum permitted dose being close to the asymptote value. Similar patterns were seen in predominantly protectant and predominantly curative control, although the range of variability between the highest and lowest levels of control was greater where control was protectant. The across experiment

mean dose response for azoxystrobin was less tightly curved, reflecting less effective control at lower doses than the other QoI active substances.

Between harvest years 2001 and 2005 a rapid decline in the control achieved was observed for all three QoI fungicides tested (Fig. 1). The pattern of decline in activity was similar in both protectant and curative situations.

3.2 Changes to the parameters of dose response curves for QoIs

The main change in the dose response curves for QoI fungicides was a decrease through time in the overall level of control that was achievable, as represented by a decrease in the asymptote of the percent control curve between 2001 and 2005 (Fig. 2). The changes in asymptotes were large in comparison with the standard errors on the parameter estimates, and regressions of asymptote on year were highly significant ($p>0.001$) in both protectant and curative situations accounting for 66.8% and 69.2% of the variation, respectively (Fig. 2). There was no significant change in the curvature parameter *k*.

3.3 DMI efficacy

Epoxiconazole showed a gradual erosion of protectant activity and a steeper decline in curative activity over the 21 years that it has been tested (Fig. 3). Prothioconazole was tested over 15 years and followed a similar pattern of decline (Fig. 4). In both actives the range between the best and worst efficacy observed within each group of seasons increased as mean efficacy declined. This was particularly evident, for example, when comparing protectant activity

between 2007-09 with activity between 2013-15, with epoxiconazole or prothioconazole. The curves denoting best and worst efficacy are fitted to data from single experiments, so the data are variable.

3.4 Changes to the parameters of dose response curves to DMI's

A significant reduction in the curvature parameter (*k*) was observed for dose responses to epoxiconazole and prothioconazole in both curative and protectant situations (Fig. 5). There was considerable variation in the parameter estimates, both within and between seasons. Nevertheless, differences between *k* values across years were large relative to the standard errors of the estimates and a regression of *k* on years was highly significant. The underlying shape of the relationship of *k* across years was difficult to discern, given the variability, however a linear model was the best fit, accounting for 38.5% of the variation ($p<0.001$, *F test*) (linear fit equation $k = 0.3122x-630.2$, where x = year). There was no improvement in explanatory power by estimating separate parameter values for the two actives or for predominantly curative or protectant applications.

The asymptotes of the response curves for both of the DMI fungicides increased as a proportion of the untreated and this change across years was significant (Fig. 6). The underlying shape of the change in asymptotes across years was uncertain due to variability of estimates, but approximated to a linear increase for both fungicides in both protectant and curative situations. The increases were different for each type of activity. Separate parallel linear regressions for protectant treatments were statistically significant: explaining 20.7% of the variation for the protectant data set ($y=0.00814x–16.13$ for

epoxiconazole, and y=0.00814x–16.20 for prothioconazole, $p = 0.012$, *F test*). In curative situations a single linear regression accounted for 35.9% of the variation (y=0.02366x-47.2, $p < 0.001$ *F test*). There was no significant improvement in explanatory power by fitting separate curves for the two products. There was indication of a curvilinear response, but this was predominantly due to one year (2008) with high leverage.

3.5 Relating *in vitro* sensitivity phenotype to field efficacy – DMI fungicides

Between 2003 and 2015 mean *in vitro* EC_{50} of epoxiconazole for populations of *Z. tritici* sampled at Rothamsted increased 17 fold from 0.042 to 0.739 micrograms per ml. The equivalent increase for prothio desthio was 20 fold from 0.006 to 0.123 (Table 3). In the two years for which data are available from a range of UK locations (2013 and 2015) EC_{50} values varied 3 or 4 fold between populations sampled from different sites (Table 3) and the Rothamsted samples in those years were well within the range observed elsewhere. The available data indicate that variation across years was substantially greater than variation across locations.

The extent to which laboratory EC_{50} values might explain field ED_{50} values was tested by regression. Laboratory EC_{50} values plotted in Fig 7 are those shown in Table 3. Field ED_{50} values were estimated from the mean dose-response curves for each year as the dose at which 50% control was obtained. Significant relationships between laboratory and field values were found for both fungicides. A regression of field on laboratory EC_{50} values for epoxiconazole was significant ($p=0.01$, *F Test, SE=0.275*) and accounted for

64.4% of the variation observed (y= 0.3128x -0.007 where y = log10 field ED_{50}, and x=log10 *in vitro* EC_{50}). For prothioconazole, the relationship was also significant (p>*0.001, F test, SE = 0.0952*) explaining 79.5% of the variation (y= 3.812x +0.246, where y= field ED_{50}, and x = *in vitro* EC_{50}). Field ED_{50} and laboratory EC_{50} values both increased in approximately year order from bottom left to top right of the scatter plots (Fig. 7). For those years when field ED_{50} values could be estimated for curative and protectant situations, the two data points are not independent of each other, as each pair of y-axis values share a common x-axis value. In each pair the dose required to achieve 50% control was higher in curative than protectant situations, but there were too few values to legitimately test whether separate regression intercepts or slopes were justified where control was predominantly curative rather than protectant.

In vitro sensitivity is usually expressed as EC_{50} in units of concentration of active substance added to agar or liquid medium. In principle, a stronger comparison of *in vitro* and field values would use concentration of active substance in leaves, so that both axes of the relationship were expressed in the same units. However, in practice values for field concentrations are difficult to quantify analytically or calculate, because the applied dose of systemic active substances (measured in grams applied per unit ground area) subsequently moves in the xylem flow and becomes diluted in the volume of the crop canopy. Field concentrations can be estimated by assuming that the proportion of the applied dose intercepted by each leaf layer within the upper crop canopy became diluted in a given leaf water content[23]. Estimates of

fungicide deposition, on a leaf area basis were provided by F. Van den Berg (pers. comm.) based on a method published previously[24]. Deposition on the upper most leaf (at the time of fungicide application) was assumed to approximate to predominantly protectant situations, and the average of two leaf layers below was assumed to approximate to deposition for curative situations. These estimated concentrations (in micrograms per ml) were used to calculate EC_{50} values for each fungicide, using the mean dose response curve for each group of years. Note that these values will be referred to as 'field EC_{50}' to distinguish them from field ED_{50} values expressed in relation to applied dose. Using this method, significant relationships were found for both fungicides (Fig 8). A regression of field on laboratory EC_{50} values for epoxiconazole was significant (*p=0.014, F Test, SE=5.24*) and accounted for 60% of the variation observed (y= 51.2x+3.88 where y = field EC_{50}, and x= *in vitro* EC_{50}). For prothioconazole, the relationship was also significant ($p<0.001$, *F test, SE = 2.49*) explaining 88.9% of the variation (y= 399.1x + 6.36, where y= field EC_{50}, and x = *in vitro* EC_{50}). In both fits the constant was not significantly different from zero. Slopes were much greater than one.

3.6 Relating genotype frequency to field efficacy – QoI fungicides

The frequency of the mutation underlying the target site G143A amino acid substitution increased rapidly from zero in 2001 and 2002 to 96% by 2006 (Table 4). A high frequency persisted to 2015. The number of isolates genotyped was particularly low in the first two years, so low levels of G143A may have been present, but not detected. Nevertheless, the frequencies reported here are similar to those reported by other workers [25] across Europe

over these critical years when there was rapid selection for insensitive strains. During the years when the population was in transition, the population distribution of sensitivities was bi-modal, comprising sensitive and insensitive sub-sets. The insensitive G143A sub-set were characterised by very high resistance factors, indicating that they would not be controllable at any legal field dose. Mean EC_{50} values are not a representative metric for bi-modal population distributions, so regression against field data was not attempted. The frequency of G143A in each year was well related to the asymptotes of the field dose response curves (Fig. 9). Regression of field dose response asymptotes (across all QoI active substances) on frequency of G143A explained 88% and 78% of the variation in asymptote in protectant and curative situations, respectively. Regression statistics for protectant data were $p<0.01$ by F Test, SE=0.091, $y=0.0066x+0.1234$, and for curative data were $p<0.01$ by F Test, SE=0.124, $y=0.0059x+0.2385$, where y=asymptote (dimensionless fraction) and x=frequency of G143A (%). Strong cross-resistance between QoI fungicides resulted in similar loss of efficacy for all active substances. Differences in asymptotes between the fungicides, observed before G143A strains became common, probably relate to differences in inherent activity of the active substances. Higher asymptotes in curative than protectant situations (Fig. 9.) were also observed before G143A became common.

4 DISCUSSION

The methodology used in the long-term run of experiments reported here ensured that the leaf layers in the canopy that were assessed were consistent

in relation to the uppermost leaf layer that was fully emerged at the time of fungicide application. This approach reduces extraneous variability between sites and seasons, enabling underlying changes in efficacy to be quantified with greater precision. The method also allowed differentiation between treatments that were predominantly controlling symptom expression from existing latent infections and treatments that were predominantly preventing new infections. This distinction can never be absolute with naturally occurring epidemics, as each leaf layer at different points in time will contain some combination of uninfected, latent, infectious and post-infectious leaf area. Nevertheless, clear differences in the performance of the fungicides were found between protectant and curative situations, particularly in the later stages of DMI insensitivity evolution, when curative efficacy deteriorated markedly.

The loss of QoI efficacy reported here is consistent with the loss reported from other parts of Europe [25] where QoI fungicides have also been widely used, resulting in selection for high frequencies of strains with the G143A cytochrome *b* mutated alleles. The reduction in DMI performance reported here for the UK and Ireland was greater than reported elsewhere in Europe.[26,27] This may be explained by differences in experimental and farm practice. Many field experiments use two or more fungicide applications to measure efficacy. In the experiments reported here, a single application was used to expose fungicides to a difficult control challenge (to better discriminate differences in performance between fungicides) and to distinguish between protectant and curative performance. Trials with multiple applications may mask an underlying deterioration in curative performance. The wet temperate

maritime climate in the UK and Ireland causes high epidemic growth rates of *Z. tritici*. This has two evolutionary consequences: high epidemic growth rates cause faster rates of selection for insensitive strains, and growers respond to high disease risk by increased fungicide use, further increasing the rate of selection.[28]

There was a different pattern in the decline in field performance of the DMIs compared to the QoI's, as expressed through changes in dose response parameters. The control achieved from the use of QoIs in the UK and Ireland reduced significantly between 2001 and 2005. This decline was well related to the increase in frequency of the G143A mutation in the UK, reported to confer a high level of insensitivity to QoI fungicides.[5] During this period the QoIs went from providing between 60% and 100% control, to less than 40% control of *Z. tritici* on average. Despite the G143A mutation being found in 90% of the UK population by 2005, some residual activity from QoIs on *Z. tritici* remained. This agrees with work showing that the QoI pyraclostrobin may still have an effect on G143A strains through a reduction in germ tube extension.[29] The results here indicate that this residual control may not be limited to just protectant activity. The G143A mutation split *Z. tritici* into two sub-populations. The wild type sub-population remained sensitive to field doses, whereas the mutant sub-population was resistant to doses above the maximum permitted field dose. The dose response asymptotes reported here were estimated from a range of field doses below those which would control the G143A sub-population. Although the asymptote, in theory, represents maximum control with infinite dose, in reality the difference between the untreated value and the asymptote represents control of the wild-type strains

and the asymptote measures a combination of the proportion of the pathogen population that carries the mutation, plus wild-type lesions which cannot be controlled because they are not in a sensitive stage of their life cycle at the time of treatment. The changes in asymptote values were a good reflection of the changes in G143A frequency.

The pattern of decline in DMIs differed substantially from that seen in the QoIs, both in relation to the speed of change in efficacy and in the dose response parameters affected. These were consistent with the effect of a succession of insensitivity changes of small effect, accumulating over many years.[30] The decline in efficacy was primarily associated with a change in dose response curvature. Mutant strains which have low or moderate resistance factors (mutant strain EC_{50} divided by wild type EC_{50}) are likely to remain sensitive to doses near the maximum permitted field dose, but be insensitive to lower doses, resulting in an observed reduction in field dose response curvature. The effect on curvature is complicated by the great diversity of insensitive variants and by variation in the deposition of dose at different points in crop canopies.[31] That the asymptote of DMI response curves was also decreasing, more especially in curative situations, suggests a proportion of the *Z. tritici* population may now be beyond control at field rates of DMI application. This agrees with the latest laboratory results showing that the most evolutionarily advanced strains of *Z. tritici*, carrying complex CYP51 variants with multiple amino acid alterations, often in combination with over-expression of CYP51 and efflux mechanisms, have DMI resistance factors of similar order to QoI resistant G143A strains.[3] These advanced strains have yet to dominate the population in the same way as QoI resistant mutants do.[12]

The balance of fitness benefit when exposed to DMI and fitness dis-benefit when not exposed, may slow the rate of displacement of more moderately insensitive strains which already dominate the population.

The impact of insensitive strains on particular parameters of dose response curves in particular situations has practical implications for growers seeking to control disease effectively. In the QoI case, the deterioration in control, at all doses in both protectant and curative situations, was so dramatic that usage against STB fell substantially and growers switched to higher use of other modes of action against STB, particularly the multi-site acting fungicide chlorothalonil.[32] QoIs remain effective against wheat rusts so retain a value in wheat fungicide programmes.

The change in DMI sensitivity, field efficacy and the resulting change in grower behaviour, was different from the QoI case. The straightening of DMI response curves caused growers to respond by using higher DMI doses to maintain control, and the reduction in curative performance resulted in more frequent applications to maximise protectant effect.[33] Whether this increase in usage was economically justified has yet to be adequately tested.

The work reported here has demonstrated, for the first time, a significant relationship between laboratory EC_{50} and field ED_{50} values for *Z. tritici* when field EC_{50} was expressed in units of proportion of maximum permitted dose. The relationship was significant despite the *in vitro* data representing the sensitivity of the *Z. tritici* population at one location (Rothamsted) and the field data representing fungicide performance against populations at multiple sites across England, Scotland and Ireland. High levels of gene flow [30] may have resulted in changes in the sensitivity of the population at Rothamsted being

broadly representative of changes in sensitivity of populations at other locations. This interpretation is supported by the range of variation in EC_{50} values between a long run of years being substantially higher than the range of variation across UK locations within a year. A persistent difference in the frequency of particular CYP51 genotypes between Ireland and the UK, did not result in the Irish field experiment data deviating notably from the general relationships between *in vitro* EC_{50} and field ED_{50}. Such a deviation may have occurred if other active substances had been tested. A wider geographic spread of samples for *in vitro* sensitivity phenotyping, and a continuous run of seasons with the phenotyping conducted to the same methods, would be desirable if resources permitted.

A significant relationship between laboratory and field EC_{50} values was also found where both axes were expressed in the same units by estimating field EC_{50} values as estimated fungicide concentration in leaves. Despite simplifying assumptions in the underlying calculation of fungicide concentrations in the crop canopy, the intercepts of the relationships were close to (and not significantly different from) the origin, which adds confidence to the predictive value – albeit that the 'prediction' was retrospective.

As might be expected, higher fungicide concentrations were required in the field than in the laboratory to achieve the same proportional level of control. For epoxiconazole and prothioconazole comparable levels of control required approximately 50 and 400 times higher concentration in the field respectively, compared to laboratory assays The high value for prothioconazole may also result from the use of the more active metabolite prothioconazole-desthio in the *in vitro* tests.[34] Other factors are: (i) all of the dose being added to liquid

medium of the laboratory assay immediately before inoculation, compared to the field situation where infection may occur before, or many days after, treatment, (ii) losses of active substance to the air and soil during field spray application, (iii) spatial variation in the applied dose to different parts of the crop canopy, and (iv) slow decay of fungicide dose *in vitro*, compared to the field where UV exposure and *in planta* metabolism increase decay.

The relationship between laboratory assays and field performance found here, supports the use of laboratory assays on new insensitive strains as an indicator of future changes in field performance. A key question is: to what extent might a relationship between laboratory assay and field performance for one mode of action be predictive for future cases of resistance against other modes of action? For example, the results from this study suggest that a change of mean laboratory EC_{50} of ten-fold or more (compared to a baseline population of sensitive wild types) has the potential to result in a practically important change in field efficacy and in the field dose required to achieve effective control. Does this imply that recently found[35] SDHI insensitive strains of *Z. tritici* with resistance factors of ten or more will impact on field performance? A point in favour of the results being generalizable is that the effect on performance depends on the insensitivity phenotype, rather than being specific to particular underlying genotypic changes or mode of action. In principle, if the baseline dose response curves for two fungicides are similar, then similar changes of sensitivity phenotype should result in similar changes to field efficacy.

A predictive difficulty remains however. New strains may demonstrate a ten- or hundred-fold shift of *in vitro* sensitivity, but it is uncertain whether they

will increase in frequency sufficiently to cause a ten- or hundred-fold shift in the sensitivity distribution of the field population. The fitness advantage over wild-type strains, which drives selection in the presence of fungicide, is indicated by the *in vitro* sensitivity of new strains. But fitness costs in the absence of fungicide, measured in the laboratory or glasshouse, have yet to be adequately associated with selection against new strains in the field. Field experiments, of the type reported here, can measure efficacy changes between years and potentially relate laboratory or glasshouse fitness measurements (either an increase in fitness in the presence of the fungicide or a decrease in fitness in the absence of the fungicide) to field efficacy.

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Table 1. Fungicides tested and included in this analysis. Other products were included in the experiments, but excluded from the long-term analysis as they were tested for insufficient years. Product names and concentrations changed over the course of the project; however, the amount of active ingredient in a full label rate of each active remained the same. a.s. is active substance. c.p. is commercial product

Active substance (a.s.)	Product name	grams of a.s. litre^{-1} c.p.	Full dose l of c.p. ha^{-1}	Company	Years in trials
epoxiconazole	Opus / Ignite	125 / 83	1.0 /1.5	BASF	1995-1998, 2001-2015
prothioconazole	Proline / Proline^{275}	250 / 275	0.8 / 0.72	Bayer	2001-2015
azoxystrobin	Amistar	250	1.0	Syngenta	1997-1998, 2001-2006
pyraclostrobin	Comet / Vivid	250	1.0	BASF	2001-2005, 2007
trifloxystrobin	Swift	500	0.5	Bayer	2001-2005

Table 2. Total number of field experiments providing dose response data from predominantly curative or protectant fungicide applications. Note that one experiment may produce both curative and protectant data, from lower and upper leaf layers in the crop canopy, respectively.

Active substance	Years reported	No. of seasons	Protectant	Curative
azoxystrobin	2001-2005	5	8	7
trifloxystrobin	2001-2005	5	19	14
pyraclostrobin	2001-2005	5	19	13
epoxiconazole	1995 -2015	19	73	49
prothioconazole	2001- 2015	15	66	40

Table 3. Mean EC_{50} values (micrograms per ml) for epoxiconazole and prothio-desthio (as a surrogate for prothioconazole) for wild type sensitive *Z. tritici,* and populations of *Z. tritici* sampled in a sub-set of years between 2003 and 2015 at Rothamsted, UK (values in rows labelled single location). In 2013 and 2015 samples were taken from 8 (2013) and 15 (2015) *Z. tritici* populations at sites across the UK. The highest and lowest mean EC_{50} values from any population sampled are given in the rows labelled multiple locations.

Active substance		Wild type	2003	2008	2012	2013	2014	2015
Epoxi	Single location	0.0029	0.042	0.101	0.396	0.276	0.311	0.739
	Highest of multiple locations					0.92		1.355
	Lowest of multiple locations					0.235		0.401
Prothio-desthio	Single location	0.0014	0.006	0.01	0.051	0.044	0.065	0.123
	Highest of multiple locations					0.145		0.267
	Lowest of multiple locations					0.036		0.081

Table 4. Frequency (%) of the G143A mutation, conferring insensitivity against QoI fungicides, in isolates of *Z. tritici* sampled at Rothamsted, UK, in a sub-set of years from 2001 to 2015. Samples were taken in consecutive years during the period of rapid selection for insensitive strains.

	2001	2002	2003	2004	2005	2006	2015
Frequency (%)	0	0	32	79	90	96	100
Number of isolates sampled (n)	9	10	90	58	48	54	53

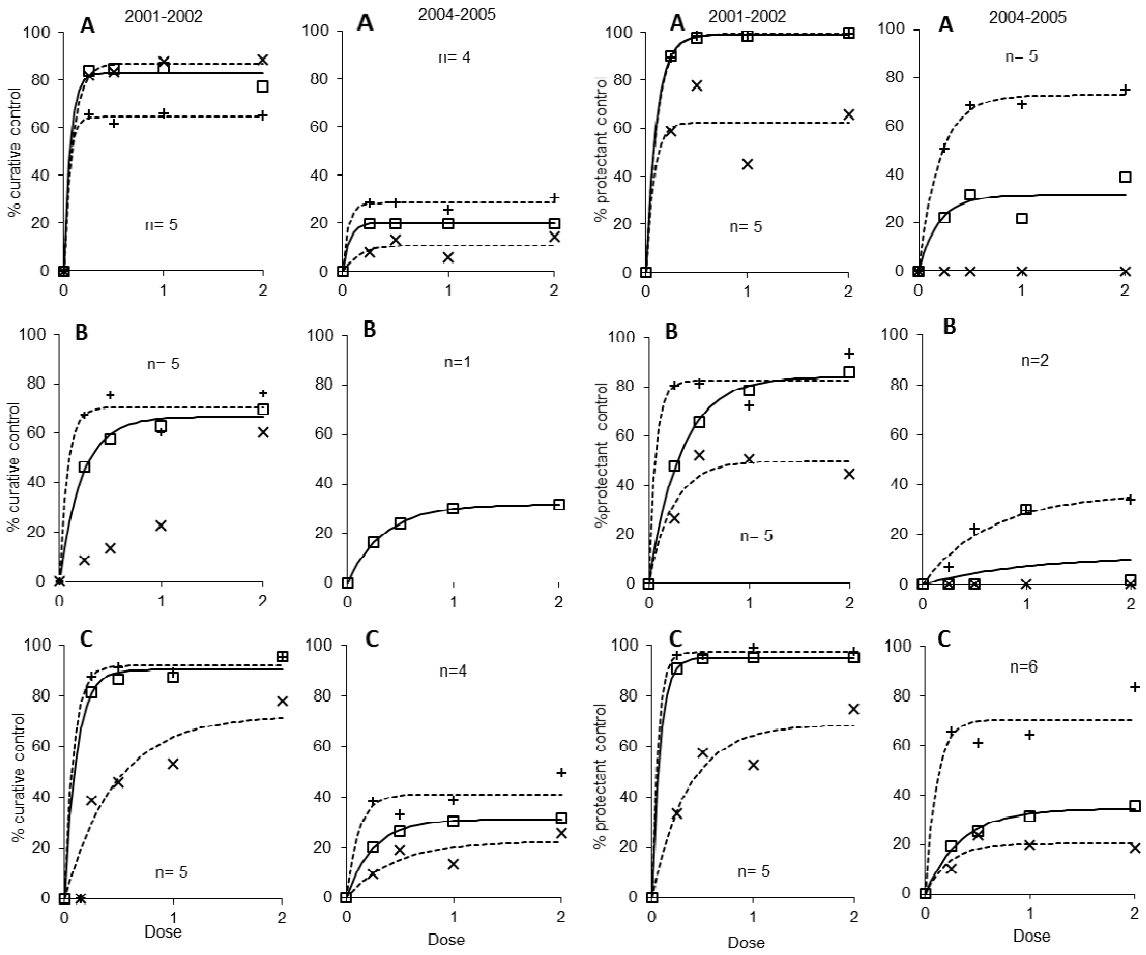

Figure 1. Comparison of QoI efficacy on *Z. tritici* in 2001-02 and 2004-2005 with three QoI fungicides trifloxystrobin (A), azoxystrobin (B), and pyraclostrobin (C) in curative (left) and protectant (right) situations. Solid lines and squares represent the mean % control achieved; dotted lines and + and x symbols represent the highest and lowest % control achieved respectively across the field experiments in each group of years. n= the number of field experiments generating dose responses within each group of years.

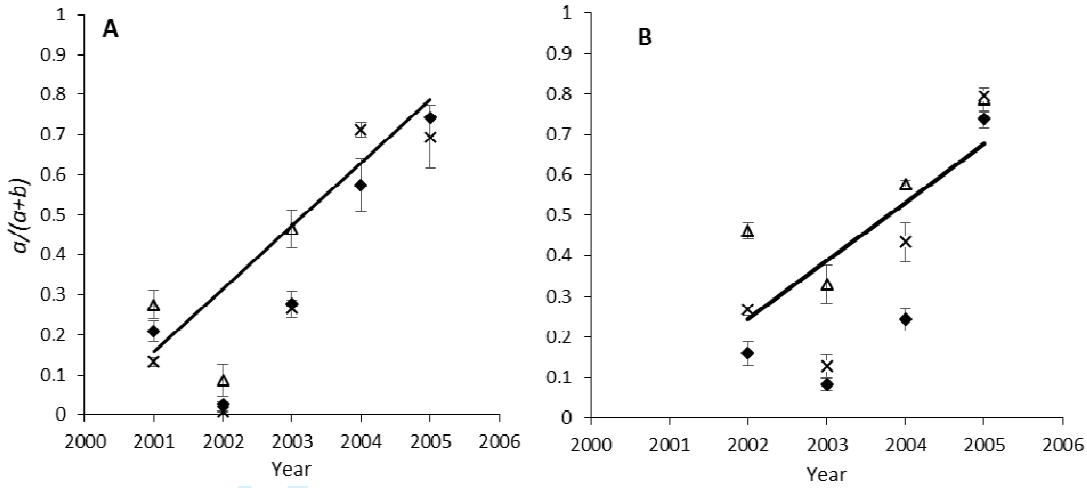

Figure 2. Changes in the asymptote (*a*) of the QoI dose response curves over time. Asymptote values represented as a proportion of untreated (*a*+*b*). A = protectant situations. B = curative situations. Symbols denote the products tested in each year. Triangles: azoxystrobin, diamonds: pyraclostrobin, crosses: trifloxystrobin. Error bars show the standard errors for the asymptote estimates for each product and year.

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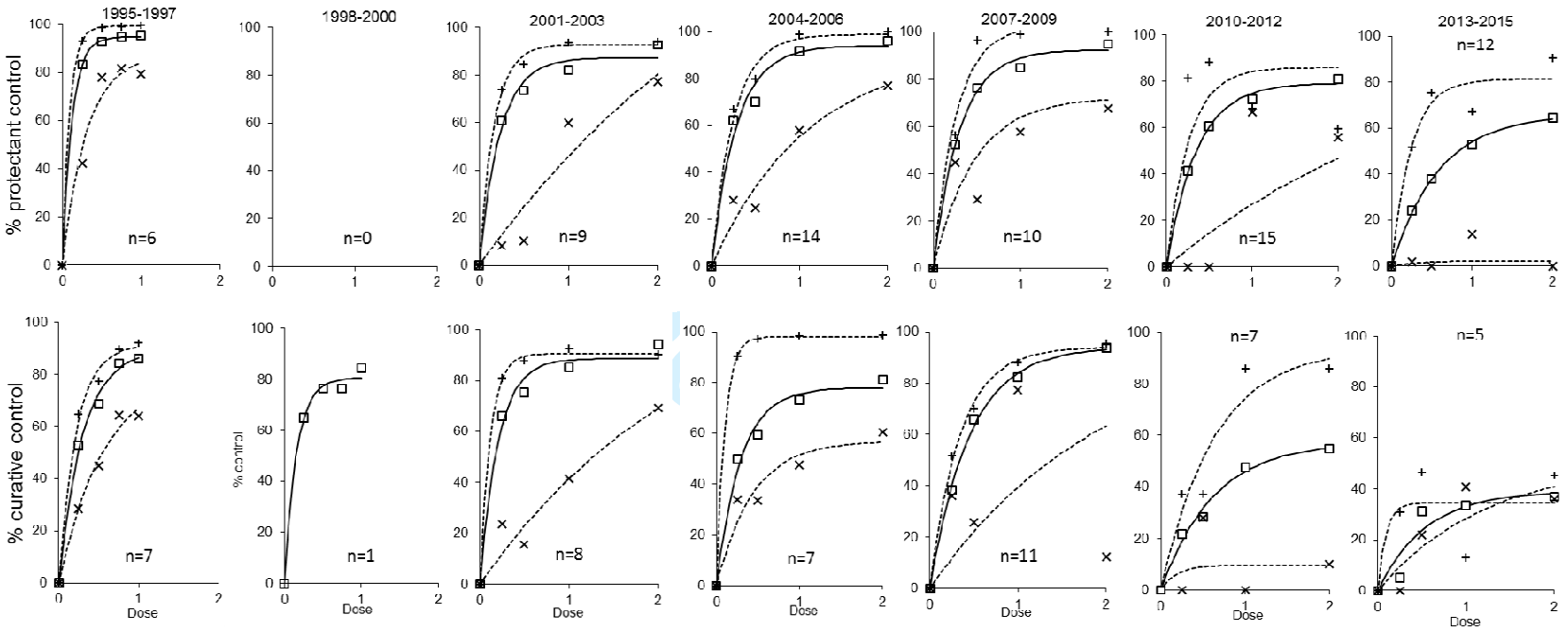
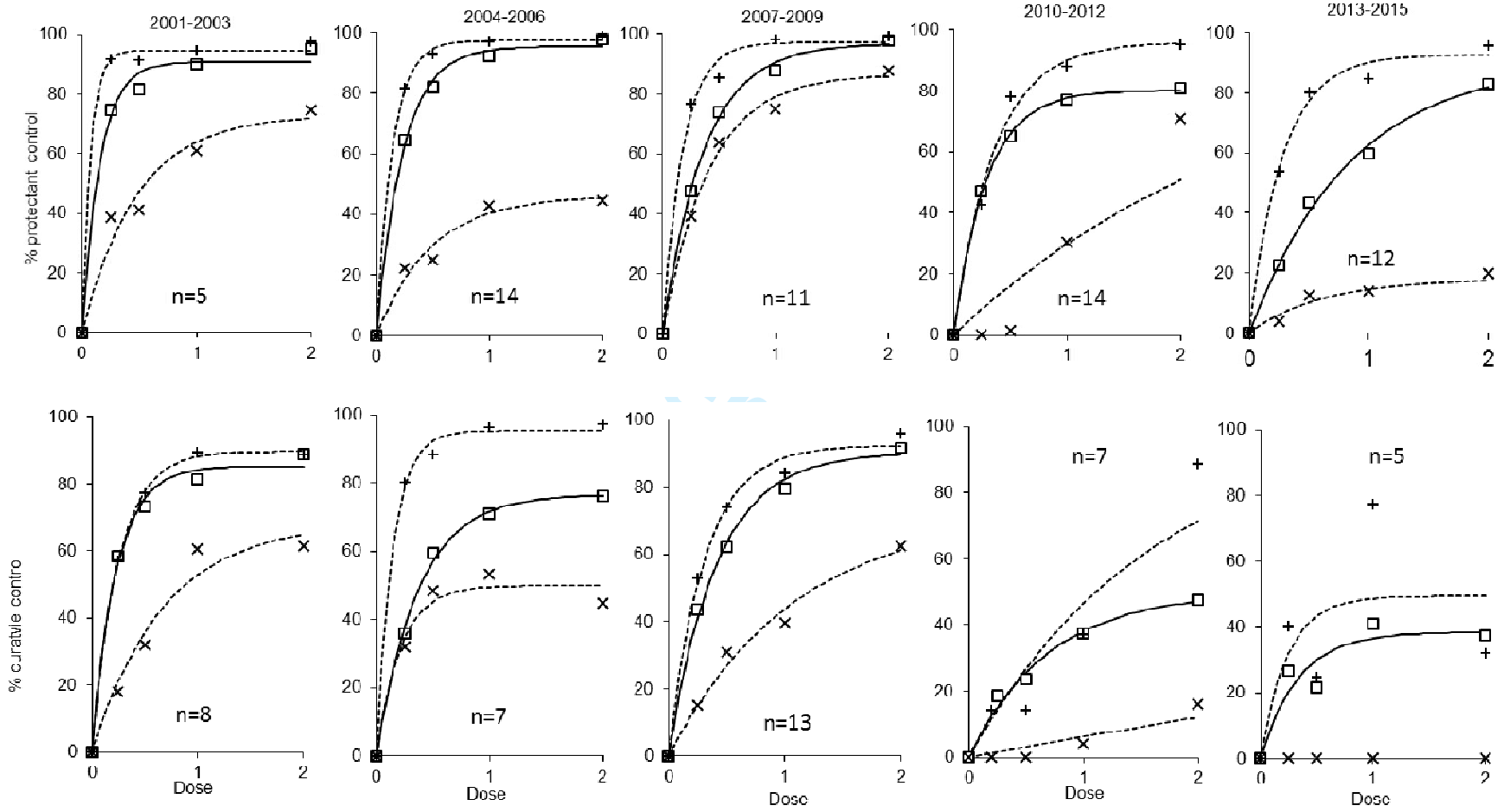


Figure 3. Percent control of *Z. tritici* with epoxiconazole in curative and protectant situations between 1995-97 and 2013-15. Solid lines represent the mean % control achieved; dotted lines the highest and lowest % control achieved across trials within each group of years. n= the number of trials generating dose responses in each group of years.


2001-2003
2004-2006
2007-2009
2010-2012
2013-2015
% protectant control
% curative control
n=5
n=14
n=11
n=14
n=12
n=8
n=7
n=13
n=7
n=5
Dose

1

1

Figure 4. Percent control of *Z. tritici* with prothioconazole in curative and protectant situations between 2001-03 and 2013-15. Solid lines represent the mean % control achieved across trials within each group of years; dotted lines the highest and lowest % control achieved and n= the number trials generating dose responses, in each group of years.

1

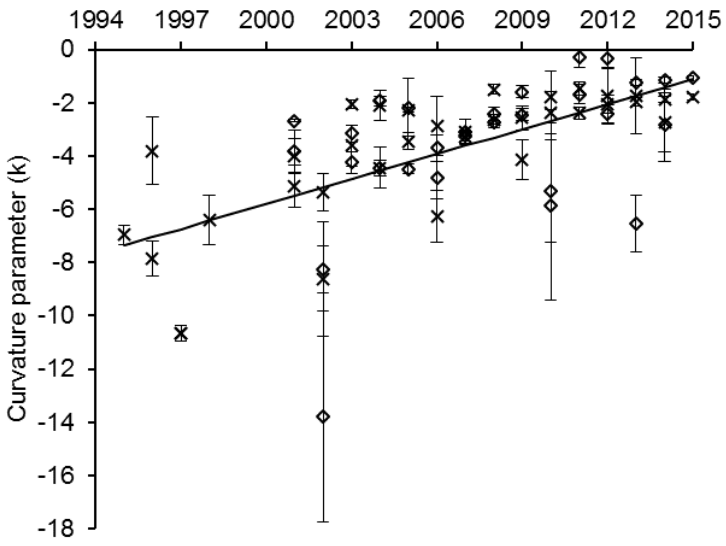


Figure 5. The change in curvature parameter estimates for protectant and curative situations between 1995 and 2015, based on mean curve fits for each year, in both situations (crosses: epoxiconazole, diamonds: prothioconazole). Error bars indicate standard errors for each product in each year

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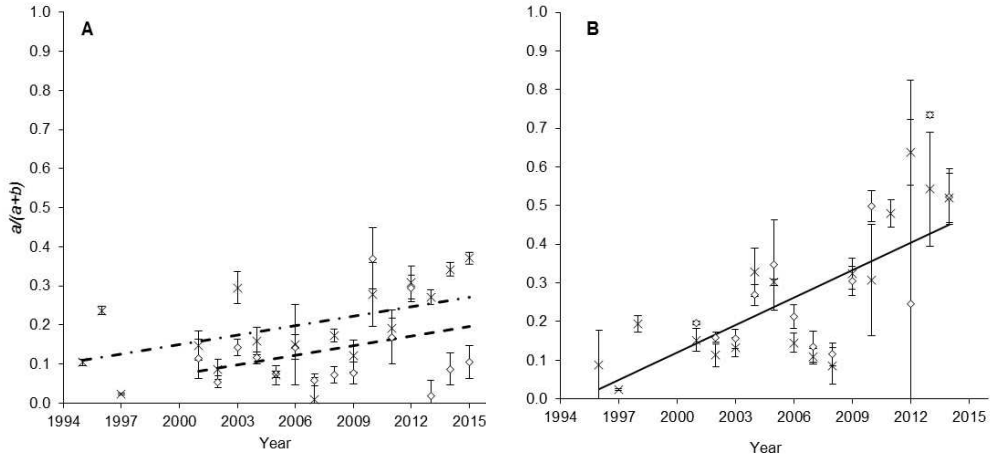

Figure 6. The increase in the asymptote (*a*) as a proportion of untreated (*a*+*b*) of dose response curves for epoxiconazole (crosses) and prothioconazole (diamonds), in protectant (A) and curative (B) situations, based on mean curve fits for each season. Dotted line shows the regression for prothioconazole, dot-dash line shows epoxiconazole in A, and solid line shows both in B. Error bars indicate standard errors.

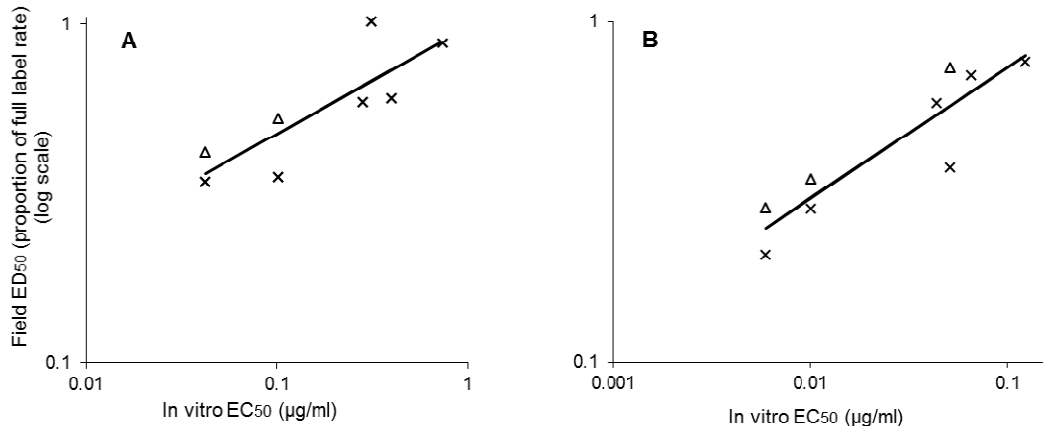

Figure 7. The relationship between mean *in vitro* EC_{50}'s and field ED_{50}'s (as a proportion of full label rate) for epoxiconazole (A), and for prothioconazole (B). *In vitro* EC_{50} tests used prothio-desthio as a surrogate for prothioconazole. Crosses: protectant field activity, triangles; curative activity. *In vitro* EC_{50}'s are available for 2003, 2008, 2012, 2013, 2014 and 2015. ED_{50} field values were not achieved at any dose in curative situations for epoxiconazole in 2012, and epoxiconazole and prothioconazole in 2013 and 2014 (no field curative activity was recorded in 2015).

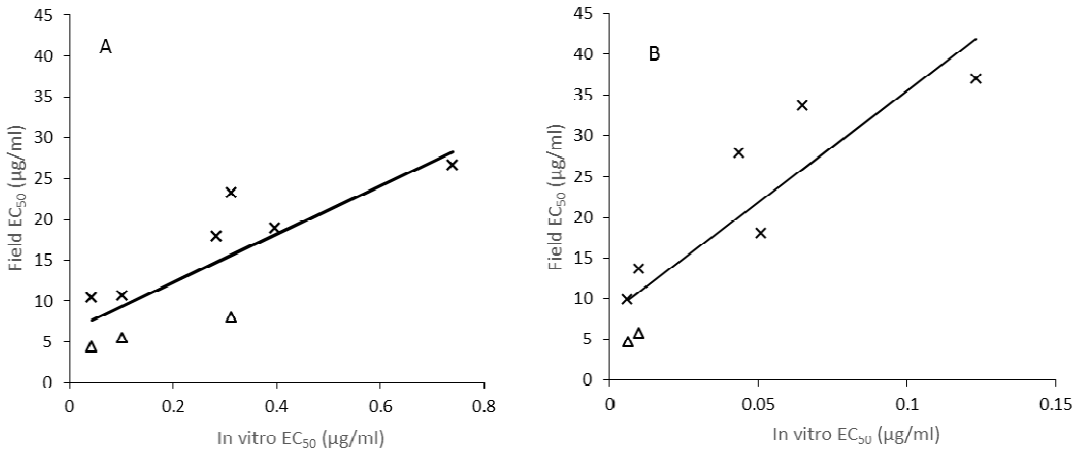

Figure 8. The relationship between mean *in vitro* EC_{50} values and estimated field EC_{50} values for epoxiconazole (A) R^2=0.63, SE=4.84, and for prothioconazole (B) with *in vitro* EC_{50} tests of prothio-desthio (as a surrogate for prothioconazole) R^2=76.6, SE 6.09. Crosses: protectant field activity, triangles; curative activity. *In vitro* EC_{50} values are available for 2003, 2008, 2012, 2013, 2014 and 2015. EC_{50} field values were not achieved (as the required level of control was not achieved) in curative situations for epoxiconazole in 2012, or for epoxiconazole and prothioconazole in 2013 and 2014 (curative activity data were not obtained in 2015).

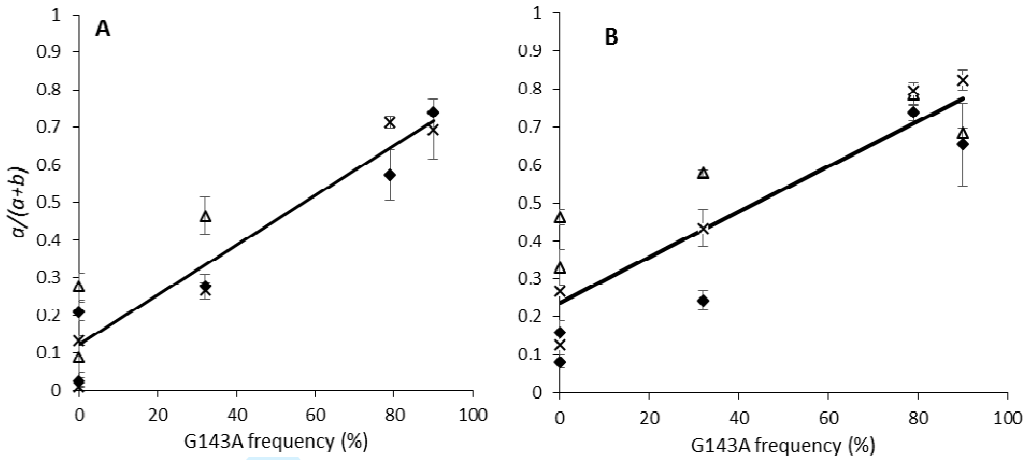

Figure 9. Relationships between asymptotes (*a*) of QoI dose response curves and G143A frequency (%) between 2001 and 2005. Asymptote values are expressed as a proportion of untreated severity (*a*+*b*). A = protectant situations. B = curative situations. Symbols denote the products tested. Triangles: azoxystrobin, diamonds: pyraclostrobin, crosses: trifloxystrobin. Error bars show the standard errors for the asymptote estimates for each product and year.

1